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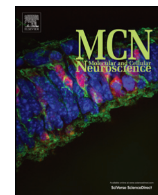

Myosin IIb controls actin dynamics underlying the dendritic spine maturation

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ABSTRACT

Precise control of the formation and development of dendritic spines is critical for synaptic plasticity. Consequently, abnormal spine development is linked to various neurological disorders. The actin cytoskeleton is a structural element generating specific changes in dendritic spine morphology. Although mechanisms underlying dendritic filopodia elongation and spine head growth are relatively well understood, it is still not known how spine heads are enlarged and stabilized during dendritic spine maturation. By using rat hippocampal neurons, we demonstrate that the size of the stable actin pool increases during the neuronal maturation process. Simultaneously, the treadmilling rate of the dynamic actin pool increases. We further show that myosin IIb controls dendritic spine actin cytoskeleton by regulating these two different pools of F-actin via distinct mechanisms. The findings indicate that myosin IIb stabilizes the stable F-actin pool through actin cross-linking. Simultaneously, activation of myosin IIb contractility increases the treadmilling rate of the dynamic pool of actin. Collectively, these data show that myosin IIb has a major role in the regulation of actin filament stability in dendritic spines, and elucidate the complex mechanism through which myosin IIb functions in this process. These new insights into the mechanisms underlying dendritic spine maturation further the model of dendritic spine morphogenesis.
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Introduction

Dendritic spines are small protrusions from neuronal dendrites. During development, spine shape changes from long, thin, headless protrusions (dendritic filopodia) to spines with short, narrow necks and large bulbous heads (mushroom spines) (Hotulainen and Hoogenraad, 2010). Most of the post-synaptic terminals of excitatory synapses reside in the dendritic spines, and the spine morphology and size modifies the synapse function. Abnormal spine shape has been detected in many neurological diseases (Calabrese et al., 2006; van Spronsen and Hoogenraad, 2010). In order to detect and understand pathogenic changes leading to neurological diseases it is fundamental to know how spines develop normally. The actin cytoskeleton is a structural element that regulates changes in spine shape as well as shape maintenance. The beauty of the actin cytoskeleton as a building element is its capacity to treadmill actin monomers through the filaments, making the actin cytoskeleton tremendously dynamic. Actin binding proteins regulate the treadmilling rate in several ways; they can facilitate the incorporation of monomers onto the barbed ends of actin filaments, protect the filaments from depolymerizing factors or increase the rate of depolymerization of actin monomers from the pointed end of the filament (Le Clainche and Carlier, 2008). Based on the treadmilling rate, actin filaments in dendritic spines can be divided into a dynamic pool (time constant < 1 min) and a stable pool (time constant ~17 min) (in addition, to an 'enlargement pool') (Honkura et al., 2008; Star et al., 2002). Synapse activity affects the proportions of the dynamic and stable F-actin pools, as well as the actin treadmilling rate (Honkura et al., 2008; Okamoto et al., 2004; Star et al., 2002). An abnormal treadmilling rate causes abnormal shape and dynamics of the dendritic spines and is a plausible cause of Baraitser–Winter syndrome (Hotulainen et al., 2009; Okamoto et al., 2007; Rivière et al., 2012).

Recently, we and others have revealed mechanisms underlying dendritic filopodia elongation as well as spine head growth from headless protrusions (Hotulainen and Hoogenraad, 2010). However, it is not known how spine heads grow further and get stabilized during dendritic spine maturation. The current view is that spine maturation does not change actin dynamics (Star et al., 2002). Taking into account that dendritic spine morphology and dynamics change during maturation (Dunaevsky et al., 1999; Oray et al., 2006; Takahashi et al., 2003), and that the actin cytoskeleton is the major structural element underlying these changes (Hotulainen and Hoogenraad, 2010), we found it

Abbreviations: DIV, days *in vitro*; FRAP, fluorescence recovery after photobleaching; MHC, myosin heavy chain; MLC_{20}, 20 kDa myosin regulatory light chain; PAGFP, Photoactivatable green fluorescent protein.

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surprising that there would be no apparent change in actin dynamics or the proportions of dynamic and stable F-actin pools.

Interestingly, we found out that there is a significant increase in the relative size of the stable F-actin pool during neuronal maturation. Furthermore, we were interested to know how F-actin stability can be regulated in dendritic spines. It is known that cofilin-1 depletion or over-expression of CaMKII can increase the relative size of the stable F-actin pool in dendritic spines (Hotulainen et al., 2009; Okamoto et al., 2007). To find other players involved in the regulation of actin filament stability, especially during spine maturation, we turned to myosin IIb which has been shown to be important for the normal development and function of dendritic spines (Hodges et al., 2011; Rex et al., 2010; Ryu et al., 2006; Zhang et al., 2005) and is re-located into dendritic spines during neuronal maturation (Ryu et al., 2006). In contrast to its recently proposed function as a de novo actin nucleator in neurons (Rex et al., 2010), we show that myosin IIb over-expression increases the relative size of the F-actin stable pool in dendritic spine heads. In line with this, myosin IIb silencing by siRNA depleted the stable F-actin pool indicating a major role in the regulation of actin filament stability. The myosin IIb motor function was not required for actin filament stabilization; in contrast, active and contractile myosin IIb increased the treadmilling rate of the dynamic F-actin pool.

Results

The relative size of the stable F-actin pool increases during neuronal maturation

To study the turnover of actin filaments in the dendritic spines of cultured neurons, we used fluorescence recovery after photobleaching (FRAP) and fluorescence decay after photoactivation (Hotulainen et al., 2009; Koskinen et al., 2012). The turnover of actin monomers in F-actin, or actin treadmilling rate is dependent on six parameters: the polymerization rates at the plus- and minus-ends, the depolymerization rates at the plus- and minus-ends, the filament length and the concentration of the free actin monomers (Halavatyi et al., 2010). In addition to this, the assembly and disassembly of the F-actin determines the rate of change in the F-actin structures. In FRAP, the treadmilling rate is estimated from the polymerization rate. In other words, FRAP assay measures how fast new actin monomers are added to the actin filament ends. And as the critical concentration is significantly lower for plus-end polymerization than for minus-end polymerization, fluorescence recovery mainly reflects to the actin polymerization in plus-ends. In PAGFP assay the estimation is done using the depolymerization rate. In other words, how fast fluorescent actin molecules dissociate from actin filaments and how fast the actin structures are disassembled. In practice, FRAP is especially useful to estimate the treadmilling rate of the dynamic F-actin pool whereas the photoactivation assay is advantageous for long recordings, and well suited to study the size and properties of the stable F-actin pool (Koskinen et al., 2012). We first recorded the fluorescence recovery after photobleaching from 14 to 21 days *in vitro* (DIV14 or DIV21) cultured hippocampal neurons (Fig. 1A and B). For these experiments, rat hippocampal neurons were transfected with a construct expressing GFP-actin one day before the imaging. Cells expressing low to moderate amounts of GFP-actin were selected for analyses. In all experiments, transfected cells grew in a dense network of neurons, ensuring the availability of a proper synaptic network. For these recordings we selected spines with similar mushroom morphology. These analyses demonstrated that the relative size of the stable F-actin pool is significantly larger in DIV21 than in DIV14 neurons (Fig. 1C). At the same time, the treadmilling rate of the dynamic actin pool is significantly faster in DIV21 than in DIV14 neurons (Fig. 1D).

To study the changes in F-actin stable pool treadmilling rate, we performed a PAGFP-actin fluorescence decay assay (Fig. 1E and F). The recordings were carried out similar to the FRAP assay, but instead of photobleaching, the PAGFP-actin was photoactivated. This allowed for longer stable recordings to obtain a reliable estimation of the F-actin stable pool size and treadmilling rate. Each single spine recording was fitted to a two-component exponential decay equation. The mean sizes of the stable F-actin pools (Fig. 1G) showed a significant increase in the relative size of the stable F-actin pool in DIV21 neurons (Fig. 1G). The photoactivation assay did not show any significant differences in the time constants of the turnover rate of the dynamic or stable pool of F-actin (Fig. 1H and I). This result differs from FRAP results. However, it is important to remember that different parameters (polymerization vs. depolymerization) are measured in these two different assays. Furthermore, it might be that time interval used during the very fast decay of the dynamic pool actin in PAGFP assay was too long to be able to distinguish between two different treadmilling rates. Thus, as the FRAP assay is more suitable to estimate the treadmilling rate of the dynamic F-actin pool, it is likely that FRAP result is closer to the truth than PAGFP result.

To confirm that our primary hippocampal neuron cultures mature as published earlier (Dunaevsky et al., 1999; Oray et al., 2006; Takahashi et al., 2003), we analyzed spine density, morphology, size and motility in DIV14 and DIV21 cultures. Shortly, hippocampal neuronal maturation was accompanied by two-fold increase in total spine density and the spine type pattern changed between DIV14 and DIV21 (fold increases: stubby 2.24; thin 1.61; mushroom 2.13) (Supplementary Fig. 1A–C). The sub-grouping of spines based on their head width and spine length showed a shift from spine head width from 0.2–0.39 μm to 0.4–0.59 μm during maturation, as well as a reduction in the relative number of very short spines (0.2–0.6 μm) and an increase in longer spines (Supplementary Fig. 1D). The comparison of the spine head width fluctuation amplitudes revealed that the DIV21 neurons were significantly less dynamic than DIV 14 neurons (71% of the DIV14 value) (Supplementary Fig. 1E–G). Together, these analyses show that our rat hippocampal neuron cultures mature as other cultures published earlier (Dunaevsky et al., 1999; Oray et al., 2006; Takahashi et al., 2003) leading to an increase in spine density, a change in spine morphology, and a decrease in spine dynamics.

Expression level of myosin IIb controls the stability of F-actin in dendritic spines

Next, we wanted to examine molecular mechanisms that could regulate actin dynamics during dendritic spine maturation. Myosin IIb has been shown to be important for the normal development and function of dendritic spines (Hodges et al., 2011; Rex et al., 2010; Ryu et al., 2006; Zhang et al., 2005) and it is re-located into dendritic spines during neuronal maturation (Ryu et al., 2006). Thus, we decided to investigate the role of myosin IIb in regulating actin in dendritic spines. A functional myosin IIb complex is comprised of three pairs of peptides: two heavy chains of 230 kDa (MHC IIb), two 20 kDa regulatory light chains (MLC_{20}), and two 17 kDa essential light chains. We first either over-expressed the myosin IIb heavy chain (MHC IIb) or inhibited the MHC IIb expression by *myh10* targeted siRNA gene silencing. The increase or decrease in the myosin IIb protein level was analyzed by myosin IIb antibody staining in cultured hippocampal neurons. On average, over-expression of mCherry-MHC IIb increased the MHC IIb expression to 360% of the intensity present in control cells (Supplementary Fig. 2A, B). In contrast, the intensity of the myosin IIb staining in MHC IIb siRNA treated cells was diminished to 35% of the intensity present in control cells (Supplementary Fig. 2A, B). We also tested if exogenous expression of the MHC IIb can rescue myosin IIb depletion by siRNA. When the cells were simultaneously transfected with MHC IIb siRNA and mCherry-MHC IIb over-expression construct, the total MHC IIb expression was on average 150% of the control cells (Supplementary Fig. 2A, B).

Next we examined whether increased or decreased myosin IIb levels affect F-actin dynamics in dendritic spines. We first performed the FRAP assay (GFP-actin) for the DIV14 neurons expressing mCherry-myosin

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